46 SCREENING FOR DRUGS USING IMMUNALYSIS DIRECT ELISA KITS

46.1 Summary

46.1.1 The Immunalysis Direct ELISA Kits are specific and sensitive in-vitro tests to detect the presence of drugs in forensic samples such as whole blood, serum, plasma and urine. The Immunalysis Direct ELISA kits consist of microplates that are coated with a polyclonal antibody with high affinity for the target analyte. These antibodies display cross-reactivity with related drugs within a drug class. An aliquot of the diluted unknown specimen is incubated with a dilution of enzyme (horseradish peroxidase) labeled drug derivative in microplate wells coated with fixed amounts of oriented high affinity purified polyclonal antibody. A competitive binding for the antibody binding sites occurs between the enzyme–labeled drug and the drug in the forensic sample. The wells are washed thoroughly to remove any unbound sample or residual reagent and a chromogenic substrate is added. The color produced is stopped using a dilute acid stop solution and the wells are read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of the drug in the sample. The results obtained are presumptive, meaning that any "positive" result requires appropriate confirmation.

46.2 Specimen Requirements

46.2.1 Approximately 100 µL of whole blood, fluid(s) or tissue dilutions/homogenates.

46.3 Reagents and Standards

46.3.1 Immunalysis Direct ELISA Kits for Amphetamine, Acetaminophen, Barbiturates, Benzodiazepines Benzoylecgonine (Cocaine Metabolite), Carisoprodol, Fentanyl, Methadone, Methamphetamine, Opiates, Oxycodone Specific, PCP, Salicylate, THC Carboxylic Acid and Zolpidem. Each kit contains:

46.3.1.1 96 well microplates coated with polyclonal antibodies.

46.3.1.2 Drug conjugate containing drug derivative labeled with horseradish peroxidase in a buffered protein solution with stabilizers (pH 7.6) containing azide free preservatives.

46.3.1.3 TMB chromogenic substrate containing 3, 3', 5, 5' tetramethylbenzidine and urea peroxidase in buffer.

46.3.1.4 Stop reagent, 1N hydrochloric acid.

46.3.2 Acetaminophen (APAP) powder

46.3.3 Amphetamine, 1 mg/mL

46.3.4 Butalbital, 1 mg/mL

46.3.5 Clonazepam, 1 mg/mL

46.3.6 Meprobamate, 1 mg/mL

46.3.7 Benzoylecgonine, 1 mg/mL

46.3.8 Fentanyl, 100 µg/mL

46.3.9 Methadone, 1 mg/mL

46.3.10 Methamphetamine, 1 mg/mL

46.3.11 Morphine, 1 mg/mL

46.3.12 Oxycodone, 1 mg/mL

46.3.13 Phencyclidine, 1 mg/mL

46.3.14 Acetylsalicylic Acid (ASA) powder

46.3.15 9-Carboxy-11-nor-delta 9-THC (THCA), 1 mg/mL

46.3.16 Zolpidem, 1 mg/mL

46.4 Solutions, Standards, Calibrators, Controls

46.4.1 Blank blood negative control (**NC**). Blood bank blood previously determined not to contain drugs.

46.4.2 Cutoff Reference Solutions:

46.4.2.1 Drugs of Abuse Cutoff Reference solution is prepared in the Central Laboratory, assigned a lot number and distributed to all 4 DFS laboratories.

46.4.2.1.1 Add the following volumes to a 100 mL volumetric flask and QS to volume with methanol

Drug	µL of 1 mg/mL standard	Final concentration (mg/L)
Amphetamine	50	0.5
Benzoylecgonine	50	0.5
Methadone	50	0.5
Methamphetamine	50	0.5
Zolpidem	50	0.5
Clonazepam	40	0.4
Morphine	40	0.4
Oxycodone	40	0.4
THCA	10	0.1
PCP	10	0.1
Butalbital	1000	10
Meprobamate	4000	40
Fentanyl	20 (100 µg/mL std)	0.02

46.4.2.4 Acetaminophen/Salicylate Cutoff Reference Solution, 0.2 mg/mL and 0.5 mg/mL respectively: Weigh 20 mg acetaminophen and 50 mg acetylsalicylic acid. Transfer to a 100 mL volumetric flask and QS to volume with methanol.

46.4.3.1.1 This solution is prepared in the Central Laboratory, assigned a lot number and distributed to all 4 DFS laboratories.

46.4.3 Blood Positive Control (**PC**)

46.4.3.1 Prepare spiked blood **PC** by adding 100 µL of Drugs of Abuse Cutoff Reference Solution and 100 µL of Salicylate/Acetaminophen Cutoff Reference Solution (as needed) to appropriately labeled tube. Dry tube under nitrogen to evaporate methanol. Reconstitute in 1 mL blank blood to prepare the following drug cutoff concentrations (mg/L). Vortex briefly.

Drug	Final Cutoff Concentration (mg/L)
Amphetamine	0.05
Benzoylecgonine	0.05
Methadone	0.05
Methamphetamine	0.05
Zolpidem	0.05
Clonazepam	0.04
Morphine	0.04
Oxycodone	0.04
THCA	0.01
PCP	0.01
Butalbital	1
Meprobamate	4
Fentanyl	0.002
Acetaminophen	20
Salicylate	50

46.4.4 Blood Low Positive Control (**LPC**)

46.4.2.4 Prepare spiked blood **LPC** by adding 50 µL of Drugs of Abuse Cutoff Reference Solution and 50 µL of Salicylate/Acetaminophen Cutoff Reference Solution (as needed) to appropriately labeled tube. Dry tube under nitrogen to evaporate methanol. Reconstitute in 1 mL blank blood. Vortex briefly. Final concentrations will be ½ the values listed in 46.4.3.1

46.4.5 Multiconstituent High Positive Control (**HPC**) provided by Immunalysis. The 4 mL aliquots of frozen blood are spiked at 2 times the final concentrations listed 46.4.3.1

46.5 Apparatus

46.5.1 Test tubes, 12 x 75 mm disposable glass

46.5.2 Vortex mixer

46.5.3 Micropipets, 8 channel multichannel pipet, pipet tips

46.5.4 Timer

46.5.5 TECAN Miniprep Micro-plate robot

46.5.5.1 Drug Screening Panels:

46.5.5.1.1 DUID Panel

Barbiturates
Benzodiazepines
Carisoprodol
Cocaine metabolite
Fentanyl
Methadone
Methamphetamine/MDMA
Opiates
Oxycodone specific
PCP

THCA
Zolpidem

46.5.5.1.2 Abused Drug Panel

Cocaine metabolite
Opiates
Oxycodone specific
Methamphetamine/MDMA
PCP

46.5.5.1.3 Full Toxicology ELISA panel

Acetaminophen
Amphetamine
Barbiturates
Benzodiazepines
Carisoprodol
Cocaine metabolite
Fentanyl
Methadone
Methamphetamine/MDMA
Opiates
Oxycodone specific
PCP
Salicylate
THCA
Zolpidem

46.5.5.2 Assays, cutoffs and volumes of diluted samples for each assay

Assay	Cutoff (mg/L)	Sample Volume (µL)
Acetaminophen	20	20
Amphetamine	0.05	40
Barbiturates	1	10
Benzodiazepines	0.04	20
Carisoprodol	4	10
Cocaine metabolite	0.05	20
Fentanyl	0.002	100
Methadone	0.05	20
Methamphetamine/MDMA	0.05	40
Opiates B	0.04	20
Oxycodone B specific	0.04	40
PCP	0.01	10
Salicylate	50	40
THCA	0.01	40
Zolpidem	0.05	40

46.5.6 Columbus Micro-plate washer

46.5.7 Magellan Micro-plate Reader

46.6 Procedure

46.6.1 Allow all blood samples and reagents come to room temperature before starting procedure.

46.6.2 Label 12 mm x 75 mm glass disposable test tubes: NC, LPC, PC, HPC and case sample IDs. Prepare controls per sections 46.4.

46.6.3 Briefly mix each sample. Pour off approximately 200 µL sample into a clean test tube (this initial step enables visualization of any clots and prevents possible cross contamination of samples with micropipette or diluter).

46.6.4 Dilute each sample 1:20 by mixing 100 µL of sample with 1.9 mL dH_2O in the appropriately labeled tubes. Smaller sample volumes (e.g., 50 µL sample diluted with 950 µL dH_2O) may be used when there is limited sample.

46.6.5 Place all diluted specimens in appropriate location on TECAN microplate robot. Select method containing the particular panel of drugs to be run on each case. When handling microplates, use caution not to touch the bottom of the microplate as this may interfere with the measurement of absorbances.

46.6.6 Create a sample dilution rack with unique ID name. Enter all sample ID's in the appropriate position with unique DFS forensic number and item number (as needed). Print sample rack list for vial verification. With every batch of samples run on TECAN robot, the identity of each diluted specimen tube is verified with the sample rack list and dilution rack location. Vial verification is documented by initials and date on the sample rack list.

46.6.7 Select method, sample dilution rack and destination plates. Start TECAN microplate robot (see TECAN miniprop operations manual for details).

46.6.8 Once TECAN miniprep robot has completed all sample and conjugate additions, remove microplates from the robot.

46.6.9 Incubate microplates for 60 minutes at room temperature in the dark.

46.6.10 Wash microplate wells 6 times with dH_2O using the microplate washer (see Columbus Microplate Washer Operations Manual for details).

46.6.11 Invert plates and slap dry on absorbent paper to ensure all residual moisture is removed.

46.6.12 Start timer. Using an 8 channel multichannel pipet, manually add 100 µL substrate to each well. Add substrate to plates in sequential order.

46.6.13 Incubate plates for 30 minutes at room temperature in the dark.

46.6.14 In the same sequential order as above, add 100 µL stop solution to each well. This will change blue color to yellow.

46.6.15 Using a dual wavelength Magellan plate reader, read absorbance of each plate within 1 hour of yellow color development (See Magellan Operations Manual for details).

46.6.16 Print ELISA drug screening report for each case file.

46.7 Calculation

46.7.1 The ratio of the absorbances of the positive controls and samples (B) relative to the negative control (B0) are multiplied by 100 to generate B/B0 values.

46.5.5.1 If the sample B/B_0 is equal to or less than the B/B_0 of the PC, the sample is presumptive positive for that class of drugs and the result is listed as "pending" for confirmation.

46.5.5.2 If the sample B/B_0 falls between the LPC and PC, the sample could contain low concentrations of drugs. The result is listed as "review" such that the toxicologist will decide whether or not to pursue confirmation of the drug depending on case history.

46.5.5.3 If the sample B/B_0 is greater than the B/B_0 of the PC, drugs were "none detected" in the sample and the result is listed as "ND."

46.8 Quality Control and Reporting.

46.4.4 The cutoff drug concentration does not change on a daily basis, but the B/B_0 associated with that cutoff may change. For QA/QC purposes, the absorbances and B/B_0 for the NC, LPC, PC and HPC are tracked (along with associated kit and control lot numbers) statewide on a daily basis. A drift in B/B_0 might indicate trends or problems such as a deteriorating control or cutoff reference solution and would require further investigation.

46.4.5 All positive results are presumptive and must be confirmed by a more specific, selective and quantitative procedure.

46.9 References

46.9.1 Immunalysis ELISA Kit Inserts, Pomona CA

46.9.2 TECAN Columbus Pro Washer Instruction Manual, 30008658 2004-12

46.9.3 TECAN Sunrise Absorbance Reader Instruction Manual, 30008746, 2005-02

46.9.4 TECAN Miniprep Logic Manual, 160013, July 1999

46.9.5 Evaluation of Immunalysis ELISA Assays for the Detection of Drugs of Abuse in Postmortem Bile and Urine. Patton, Isenschmid, Helpler and Schmidt. SOFT Annual Meeting, Portland, OR 2003.

46.9.6 Evaluation of Immunalysis ELISA Assays for the Detection of Drugs of Abuse in Postmortem Blood. Isenschmid, Patton, Helpler and Schmidt. TIAFT Annual Meeting, Melbourne, Australia 2003.

46.9.7 Validation of the Immunalysis Microplate ELISA for the Detection of Buprenorphine and its Metabolite Norbuprenorphine in Urine. Miller, Torrance and Oliver. JAT 30: 115-119, March 2006.

46.9.8 Validation of the Immunalysis Microplate ELISA for the Detection of Methamphetamine in Hair. Han, Miller, Lee, Park, Lim, Chung, Wylie and Oliver. JAT 30: 380-385, July 2006.

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